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A rapid reproducible test for determining rabies neutralizing antibody *

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Rabies neutralizing antibody levels in human and animal sera were tested by a rapid fluorescent focus inhibition technique, in which BHK-21 cells were infected with tissue-culture-adapted rabiesvirus. The results, obtained in 24 hours, were comparable with those of the standard mouse neutralization test.

Webster & Dawson (1935) devised an *in vivo* test for determining rabiesvirus neutralization (VN) antibodies: dilutions of sera are mixed with a constant virus dose, incubated for 1½ hours, and inoculated intracerebrally into weanling mice. This has been the accepted and most common procedure for testing rabies VN antibody (Atanasiu, 1966), although it is an expensive one with the inherent disadvantage of a 2-week interim between inoculation and final results. Moreover, variability in mouse susceptibility may affect the results (Johnson & Leach, 1940).

A cytopathic effect with rabiesvirus, although reported by some authors (Atanasiu & Lepine, 1959; Fernandes et al., 1963; Kissling & Reese, 1963; Abelseth, 1964; Wiktor et al., 1964; Johnson, 1969), has not been routinely reproducible, and the tissue culture (TC) neutralization techniques used in other viral diseases, such as metabolic inhibition in poliomyelitis (Salk et al., 1954) or cytopathic effect in monolayer for measles (Enders & Peebles, 1954), have not been readily applicable to this disease. In recent years, however, several laboratories have developed *in vitro* techniques for rabies VN testing. The California State Department of Health currently uses a fluorescent focus inhibition test in which a mixture of low egg passage (LEP) Flury rabiesvirus and the serum to be tested is added to a baby hamster kidney (BHK) cell monolayer, and the resultant presence or absence of viral invasion is examined by fluorescent antibody (FA) staining (Lennette & Emmons, 1971). Similar tests using Challenge Virus Standard (CVS) fixed virus on chick fibroblast (King et al., 1965) and the ERA strain of rabiesvirus on BHK-21 (Debbie et al., 1972) have been described.

The US Public Health Service Advisory Committee on Immunization Practices (1972) recommends that " all who receive . . . pre-exposure vaccination should have serum tested for neutralizing antibody " (a similar recommendation has been made by the WHO Expert Committee on Rabies, 1972), and our laboratory thus receives approximately 5 000 sera for VN testing every year. Working along lines similar to those of the California State Department of Health, we have developed a rapid fluorescent focus inhibition test (RFFIT) that requires only 24 hours for completion; the results of this test correlate very well with those of the mouse neutralization test in measuring VN antibody levels. This paper describes this rapid technique, which appears to be at least as sensitive and reproducible as the mouse VN test.

MATERIALS AND METHODS

Sera

Human sera routinely received in this laboratory from state health departments and sera from foxes, dogs, skunks, racoons, mongooses, cats, and monkeys used in experimental studies in this laboratory were tested by VN and by the RFFIT. All sera were inactivated at 56°C for 30 min prior to testing. Highly contaminated sera, sera held for longer than 6 months, and animal sera [1] used in the RFFIT were

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[1] Approximately 10% of all normal animal sera show evidence of nonspecific inhibition of virus at dilutions up to 1 : 5; all sera from unvaccinated animals or animal sera with expected titres of under 1 : 5 should thus be pretreated with kaolin.

treated with kaolin to adsorb nonspecific inhibitors (Hierholzer et al., 1969). An equal volume of 25% kaolin was added to each serum, and the mixture was allowed to stand at room temperature for 20 min with frequent agitation; it was then centrifuged at 1 800 rev/min for 10 min at room temperature. The supernatant fluid was considered to be a 1 : 2 dilution of the original serum. NIH reference serum lot No. 4 (1971-10-07) was used as a positive control. A pool of unvaccinated human sera was used as a negative control.

Virus

The CVS-11 strain of rabiesvirus (Kissling, 1958) was used to prepare a pool of virus in BHK-21 13s cells. After 8 passages in BHK-21 13s, it had a weanling mouse intracerebral LD_{50} of $10^{6.0}$/0.03 ml. A 1 : 200 dilution containing 16 000 PFU/0.1 ml gave good cell infectivity at 24 hours, as indicated by FA staining; from titrations with NIH reference serum, this was determined to be the optimum challenge dose.

Tissue culture

BHK-21 clone 13s cultures were grown in 250-ml plastic flasks.[1] The growth medium was Eagle's minimal essential medium as modified by MacPherson & Stoker (1962) supplemented with 10% inactivated fetal calf serum and 10% tryptose phosphate broth. BHK monolayers 1–4 days old (between the 25th and 50th passage levels) were trypsinized for use in the test. Suspensions containing 1×10^5 cells per 0.2 ml of growth medium were added to each of the 8 chambers of a Lab-Tek TC chamber slide [2] (Fig. 1).

DEAE-dextran treatment

A stock 1% solution of DEAE-dextran [3] was prepared in distilled water and kept under refrigeration. The cells were treated for 10 min at room temperature with a solution of DEAE in growth medium at a final concentration of 10 μg/ml immediately before their use in the RFFIT.

Mouse VN test

The VN test was performed in weanling mice (5 mice per dilution of serum) according to the standard procedure recommended by WHO (Atanasiu, 1966). Human sera were tested at 1 : 5 and 1 : 50 dilutions (screening dilutions); for more exact quantification of antibody titres, 5-fold serum dilutions were made. All sera were tested against 10–100 $MICLD_{50}$/0.03 ml of CVS 27.

RFFIT

The same serum dilutions used in the VN test were made in Lab-Tek TC chamber slides for the RFFIT. An equal volume of challenge virus was added to all serum dilutions, and Lab-Tek slides were then incubated at 35°C in a controlled humidity carbon dioxide chamber for 90 min. A suspension of 1×10^5 cells in 0.2 ml of growth medium was then added to each of the 8 slide chambers, and the slide was returned to the carbon dioxide incubator.

FA reading

After a 24-h incubation period, the growth medium was removed and the slides were rinsed once in phosphate-buffered saline and once in acetone at 4°C, and then fixed in acetone at −20°C for 5 min. The FA staining (Goldwasser & Kissling, 1958) was performed as described by Dean (1966), with conjugate prepared in this laboratory according to standard procedures (Levy & Sober, 1960; Marshall et al., 1958), and absorbed twice against normal BHK-13s cells. The slides were examined with a Zeiss microscope using a UG 1 exciter filter and No. 65 and 41 barrier filters, and illuminated with an OSRAM HBO 200 high pressure mercury vapour lamp. Twenty low-power (160×) microscopic fields were observed for each dilution chamber, and the numbers of fields containing fluorescing cells were tabulated. Of the 20 fields observed in the challenge virus control slides, 18–20 contained fluorescing cells (Fig. 2). A reduction of 50% or more in the number of fields with fluorescing cells (Fig. 3) was considered indicative of neutralizing antibody in the serum tested.

RESULTS

Table 1 compares the titres obtained by both VN and the RFFIT on 512 sera from persons receiving pre- or post-exposure rabies immunization. It can be seen that there was complete correlation in 487 of the 512 sera—either positive by both tests or negative by both. With 25 sera, however, the RFFIT was positive but the VN was negative, suggesting that, at least with these human sera, the test is as sensitive as (and apparently a little more sensitive

[1] Falcon Plastics, Division of BioQuest, Los Angeles, Calif., USA.

[2] Lab-Tek Products, Division of Miles Laboratories, Inc., Westmont, Ill., USA.

[3] Pharmacia, Uppsala, Sweden.

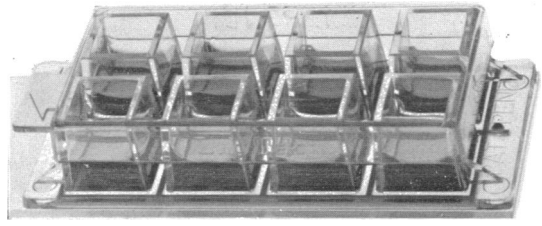

Fig. 1. Lab-Tek chambers used in the rapid tissue culture neutralization test

Fig. 2. Results of negative serum in rapid tissue culture neutralization test

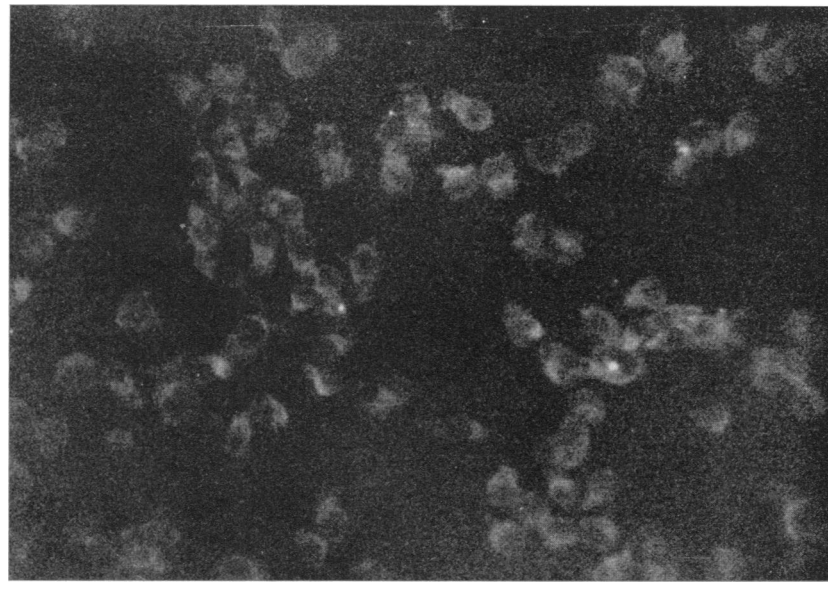

Fig. 3. Results of positive serum in rapid tissue culture neutralization test

Table 1. Correlation of the results of antibody determinations on 512 sera by the RFFIT and mouse VN tests

Result		No. of sera
RFFIT	VN	
+	+	366
−	−	121
+	−	25
−	+	0

Table 2. Variation in the number of fluorescing foci in RFFIT using standard virus dose [a] and varying dilutions of NIH reference serum [b]

Test	Serum dilution		
	1 : 320	1 : 160	1 : 80
1	20/20	8/20	0/20
2	20/20	12/20	0/20
3	18/20	13/20	0/20
4	20/20	13/20	0/20
5	20/20	20/20	0/20
6	20/20	9/20	0/20
7	20/20	14/20	0/20

[a] 0.16 PFU CVS-11/BHK 13s cell.
[b] NIH serum, lot 4 (1971-10-07), VN titre 1 : 200.

Table 3. Variation in the number of fluorescing foci in the RFFIT using standard virus dose [a] and varying dilutions of two human sera [b]

Serum No.	Test No.	Serum dilution	
		1 : 5	1 : 50
1	1	0/20	20/20
	2	0/20	20/20
	3	0/20	16/20
	4	0/20	18/20
2	1	0/20	20/20
	2	0/20	20/20
	3	0/20	20/20
	4	0/20	20/20

[a] 0.16 PFU CVS-11/BHK 13s cell.
[b] Both gave titres of 1 : 16 by the VN test.

Table 4. Representative antibody titres determined by virus neutralization and rapid fluorescent focus inhibition technique

Patient/ animal No.	Serum (S) or cerebrospinal fluid (CSF)	Day	Titre	
			VN	RFFIT
		Human cases		
1	S	13	1 : 18	1 : 25
	CSF	13	<1 : 2	<1 : 2
	S	17	1 : 230	1 : 280
	S	18	1 : 480	1 : 300
	S	19	1 : 1 400	1 : 800
	CSF	19	1 : 18	1 : 11
	S	25	1 : 1 750	1 : 1 800
	S	29	1 : 2 400	1 : 1 800
	CSF	29	1 : 95	1 : 56
2	S	5	<1 : 2	<1 : 5
	S	6	1 : 5	1 : 11
	CSF	6	<1 : 2	<1 : 5
	S	7	1 : 33	1 : 11
	S	8	1 : 18	1 : 11
		Animals vaccinated against rabies		
monkey 1	S		1 : 9	1 : 11
monkey 2	S		1 : 95	1 : 56
monkey 3	S		1 : 95	1 : 56
monkey 4	S		1 : 95	1 : 56
fox 1	S		1 : 160	1 : 125
dog 1	S		1 : 33 000	1 : 40 000
dog 1	CSF		1 : 4 800	1 : 2 400
dog 2	S		<1 : 5	<1 : 5
dog 3	S		<1 : 5	<1 : 5
dog 4	S		1 : 11	1 : 11
dog 5	S		<1 : 5	1 : 11
dog 6	S		<1 : 5	<1 : 5
dog 7	S		1 : 20	1 : 20
dog 8	S		<1 : 5	<1 : 5
dog 9	S		1 : 25	1 : 20
dog 10	S		<1 : 5	<1 : 5

than) the VN test since the majority of these 25 were positive by VN when tested at a 1 : 2 dilution. None of the sera tested was positive by the classical mouse VN test but negative by the RFFIT, indicating that

the dose of CVS-11 challenge virus/BHK cell (in the RFFIT) resulted in a test system comparable with the VN in mice for detecting low levels of neutralizing antibody. The latter result is further supported by the fact that none of 100 pre-immunization human sera tested by the RFFIT was positive. The RFFIT gave negative results with 20 normal sera from foxes, racoons, skunks, opossums, cats, mongooses, and dogs, indicating that the same sensitivity and specificity can be expected with animal sera.

Table 2 shows the results of 7 RFFIT tests run to verify the reproducibility of the results obtained when a standard virus challenge dose/cell is used. The minimum number of fluorescent foci seen in the 1 : 160 serum dilution of the NIH Reference serum (titre 1 : 200 by the VN test) was 8/20 and the maximum was 20/20 (mean, 11/20). Table 3 shows the same reproducibility of titre determination when two human sera with titres of 1 : 16 by the VN test were tested by the RFFIT—the titres were comparable in each test run.

Table 4 shows the antibody levels of representative human and animal sera examined by both tests. It is again to be noted that the titres are quite comparable, the great majority of sera being positive or negative by both tests; one serum (dog No. 5) was positive by the RFFIT but negative by the VN test, but no serum was positive by VN and negative in the RFFIT.

DISCUSSION

Sternberg (1892) demonstrated that humoral antibody capable of neutralizing the infectious capacity of a viral agent is present after a man or an animal has recovered from an overt or inapparent viral infection. The measurement of these neutralizing antibodies in the serum is direct and is one of the most biologically significant methods of determining resistance to infection. Dean et al. (1964) showed that only 3 (4.7%) of 64 vaccinated dogs with detectable serum antibody succumbed to rabies on challenge, while 19 (90.5%) of 21 control dogs and 63 (64.3%) of 98 of vaccinated dogs with no detectable antibody died. The mouse VN test measures the ability of serum antibody to prevent the lethal infection of mice with a mouse-adapted CVS rabiesvirus. The RFFIT measures the ability of serum antibody to block the infection of BHK-21 cells with a BHK-21 adapted CVS rabiesvirus, and thus is an *in vitro* technique that measures essentially the same biological factor present in the *in vivo* test currently accepted as the standard. The close correlation between the RFFIT and VN test results indicates that neutralizing capacity was being measured, rather than some undefined factor such as that measured in the indirect fluorescent antibody (Goldwasser & Kissling, 1958), haemagglutination inhibition (Halonen et al., 1968), immune lysis (Wiktor et al., 1968), and passive haemagglutination (Gough & Dierks, 1971) tests.

FA staining is a reliable indicator of the presence or absence of rabiesvirus in cell culture (Kaplan et al., 1960). The addition of DEAE-dextran reduces to 18 h the time at which fluorescence can first be observed in rabiesvirus-infected BHK cells, and it also reduces the virus dose necessary to infect those cells (Kaplan et al., 1967). At 24 h 25–50% of DEAE-treated BHK-21 13s cells infected with approximately 0.3 PFU/cell CVS 11 rabiesvirus show the " bright fluorescence, more than 50 medium and large granules " previously described by other workers (Kaplan et al., 1967). The results of the RFFIT can be easily read at 24 h, compared with 5–6 days for the plaque neutralization technique (Sedwick & Wiktor, 1967).

We are at present adapting the RFFIT procedure to microtitre II TC plates [1] and in turn to the automatic microtitre equipment [2] used for routine HI testing.

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[2] Cooke Engineering, Roanoke, Va., USA.

RÉSUMÉ

ÉPREUVE RAPIDE ET REPRODUCTIBLE POUR LE TITRAGE DES ANTICORPS NEUTRALISANTS ANTIRABIQUES

Diverses épreuves *in vitro* ont été récemment mises au point en vue de remplacer l'épreuve de neutralisation chez la souris pour le titrage des anticorps neutralisants antirabiques mais elles ont le désavantage d'exiger de 4 à 7 jours pour leur exécution.

Les auteurs décrivent un test rapide (24 heures) basé sur l'inhibition de l'invasion de cellules BHK-21 par un virus rabique d'épreuve, décelée par l'immunofluorescence. Les résultats obtenus par cette technique présentent une concordance très satisfaisante avec ceux de l'épreuve de

neutralisation chez la souris. Sur 512 sérums humains testés par l'une et l'autre méthodes, 487 étaient soit positifs soit négatifs dans les deux cas, 25 étaient positifs en épreuve d'inhibition et négatifs en épreuve de neutralisation chez la souris, et aucun sérum n'était négatif en inhibition et positif en neutralisation. Des épreuves d'inhibition pratiquées sur 100 sérums de sujets non vaccinés et sur 20 sérums normaux de chiens, de chats, de mangoustes, de mouffettes, de ratons laveurs et d'opossums ont donné des résultats négatifs.

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